methods

Serum nonesterified fatty acids have utility as dietary biomarkers of fat intake from fish, fish oil, and dairy in women[S]

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Abstract **Nutritional studies rely on various biological specimens for FA determination, yet it is unclear how levels of serum NEFAs correlate with other circulating lipid pools. Here, we used a high-throughput method (<4 min/sample) based on multisegment injection-nonaqueous capillary electrophoresis-mass spectrometry (MSI-NACE-MS) to investigate whether specific serum NEFAs have utility as biomarkers of dietary fat intake in women. We first identified circulating NEFAs correlated with long-term/habitual food intake among pregnant women with contrasting dietary patterns (n = 50). Acute changes in serum NEFA trajectories were also studied in nonpregnant women (n = 18) following high-dose (5 g/day) fish oil (FO) supplementation or isoenergetic sunflower oil placebo over 56 days. In the cross-sectional study, serum ω-3 FAs correlated with self-reported total ω-3 daily intake, notably EPA as its NEFA (r = 0.46; P = 0.001), whereas pentadecanoic acid was associated with full-fat dairy intake (r = 0.43; P = 0.002), outcomes consistent with results from total FA serum hydrolysates. In the intervention cohort, serum ω-3 NEFAs increased 2.5-fold from baseline within 28 days following FO supplementation, and this increase was most pronounced for EPA (P = 0.0004). Unlike for DHA, circulating EPA as its NEFA also strongly correlated to EPA concentrations measured from erythrocyte phospholipid hydrolysates (r = 0.66; $P = 4.6 \times 10^{-10}$) and was better suited to detect dietary nonadherence. We conclude that MSI-NACE-MS offers a rapid method to quantify serum NEFAs and objectively monitor dietary fat intake in women that is complementary to food-frequency questionnaires.**—Azab, S. M., R. J. de Souza, K. K. Teo, S. S. Anand, N. C. Williams, J. Holzschuher, C. McGlory, S. M. Philips, and P. Britz-McKibbin.

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The accurate assessment of dietary fat intake remains a methodological challenge, reflecting decades of conflicting evidence regarding the benefits of a low-fat diet for public health (1). Validated semiquantitative food-frequency questionnaires (FFQs) are widely used dietary assessment tools in large-scale observational studies because they can reliably differentiate habitual dietary patterns and estimate micro- and macronutrient intake in a cost-effective manner (2). However, FFQs are prone to recall bias, errors in estimation of true portion sizes, and selective reporting (3); this problem is exacerbated when assessing habitual fat intake due to the large variation of FA species in the diet and the tendency for underreporting fat consumption (4). Errors associated with participant self-reporting have been recognized as one of the greatest obstacles in nutritional epidemiology, limiting our ability to capture food exposures in contemporary societies (5). Comprehensive

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Abbreviations: CE, capillary electrophoresis; CV, coefficient of variation; DQI, diet quality index; FAMILY, Family Atherosclerosis Monitoring in Early Life; FFQ, food-frequency questionnaire; FO, fish oil; MSI-NACE-MS, multisegment injection-nonaqueous capillary electrophoresis-mass spectrometry; MTBE, methyl-*tert*-butyl ether; OCFA, odd-chain fatty acid; PL, phospholipid; QC, quality control; RMT, relative migration time.

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metabolite profiling (i.e., metabolomics) offers a strategy to objectively measure complex dietary patterns, including the discovery of new biomarkers of recent food intake (6). An optimal dietary biomarker is readily measurable in a minimally invasive human biofluid (urine or blood), specific to a single food group (selective), responsive to changes in the amount of food consumed (dose response) over a desired time frame (time response), and is ideally neither generated in vivo nor extensively biotransformed (exogenous) (7). For example, proline betaine is a reliable dietary biomarker of recent citrus intake (<24 h) in plasma and urine samples that has been validated in several independent observational and intervention studies (3, 8–10). Such biomarkers generally do not exist for most FAs because they are synthesized de novo from carbohydrates and other FA precursors; however, there are some exceptions, such as certain PUFAs, odd-chain FAs (OCFAs), and *trans*-FAs, which are primarily derived from specific food sources and thus largely not synthesized in vivo (11).

There is an evolving consensus regarding optimal nutritional guidelines for dietary fat, with greater emphasis placed on assessing the specific type of fat within complex dietary patterns as opposed to measuring total fat intake and single nutrients (12). For example, many jurisdictions have now restricted industrial *trans*-FA exposures from processed foods due to their deleterious effects on cardiovascular health compared with nonrestricted populations (13). Recent findings from the 21-country Prospective Urban and Rural Epidemiological study reported that total fat is correlated with lower total mortality and increased saturated fat or total dairy consumption with a lower risk for cardiovascular events (14, 15); however, these studies have relied on estimating intakes of FAs from FFQs and gathered health information from different countries with the potential for residual confounding. High-throughput methods for objective measurements of circulating FA concentrations are thus needed to provide a more standardized approach for the accurate assessment of habitual fat consumption. This is important given conflicting data regarding the putative health benefits of dietary intake of essential ω-3 PUFAs (16), such as clinical trials involving supplementation of EPA and DHA from fish oil (FO) or a prescription-based EPA analogue for primary or secondary prevention of cardiovascular disease events (17, 18). Various biospecimen types have been used for FA determination, and each reflects a different time interval associated with dietary fat intake, including adipose tissue (1–2 years), erythrocyte membrane (2–3 months), serum phospholipids (PLs) or cholesteryl esters (past few days), and TG (past few hours) fractions (4, 11, 19). While adipose tissue may be useful for the long-term assessment of dietary fat intake, such biopsies are invasive and cannot be routinely collected. In addition, erythrocytes isolated from whole blood are prone to hemolysis during processing and long-term storage (20, 21), and they are not often available in most biobank repositories, unlike serum or plasma. As of 2016, it was reported that 90% of studies analyzing ω-3 PUFAs used erythrocyte membrane PLs, serum PLs, or total (hydrolyzed) plasma lipids (22). Alternatively, fasting serum NEFAs are a more accessible protein-bound lipid pool that is released into the circulation by the hydrolyzing action of lipases on TGs from adipose tissue (4). Nevertheless, there have been few reports to date examining the utility of NEFAs as biomarkers of dietary intake in nutritional studies compared with total FAs from serum extracts and other blood fractions or adipose tissue samples (19, 23). For instance, GC methods allow for the high-efficiency separation of FAs and their isomers (24) but are limited by long analysis times and precolumn chemical derivatization procedures that contribute to bias due to the hydrolysis of esterified FAs from other lipid classes, impeding reliable serum NEFA determination (25).

Herein, we performed two studies to assess the utility of serum NEFAs as convenient dietary biomarkers of fat intake in women when using multisegment injection-nonaqueous capillary electrophoresis-mass spectrometry (MSI-NACE-MS). This multiplexed separation method offers higher sample throughput (<4 min/sample) and stringent quality control (QC) for the direct analysis of NEFAs from serum extracts without fractionation, hydrolysis, and precolumn chemical derivatization, unlike conventional GC methods (26). For the first time, we assess a cross-section of pregnant women with contrasting diet quality patterns (27) when using MSI-NACE-MS to identify specific serum NEFAs that serve as biomarkers of habitual intake of fish/seafood, full-fat dairy products, and fiber compared with FAs from total serum hydrolysates. In addition, time-dependent changes in serum EPA and DHA in young women participating in a placebo-controlled, repeated-measures trial of high-dose FO supplementation (28) were also examined as their circulating NEFAs relative to independent erythrocyte membrane PL measurements.

MATERIALS AND METHODS

Serum NEFA biomarkers of dietary fat intake in pregnant women from FAMILY

The Family Atherosclerosis Monitoring in Early Life (FAMILY) study is a prospective birth cohort study involving 839 predominantly White European pregnant women recruited from the greater Hamilton area in Ontario, Canada, between 2002 and 2009 (27). Fasting blood samples were collected in the second trimester, and serum was fractionated within 2 h from collection according to standard protocols and stored at −80°C. Comprehensive clinical and dietary data from all participants were also collected. Informed consent was obtained from all participants, and research ethic boards from Hamilton Health Sciences, St. Joseph's Health Centre (Hamilton), and Joseph Brant Memorial Hospital (Burlington) approved this protocol that abides by the Declaration of Helsinki principles. In this study, we used purposive sampling, after excluding smokers and women with gestational diabetes mellitus, to generate a subset of women (n = 50) from 226 eligible participants, half of whom consumed a healthy diet and half of whom consumed a poor-quality diet as assessed by a diet quality index (DQI) score (29), with a mean age of 32 years (range: 17–43 years) and prepregnancy BMI of 27 kg/m^2 (range: 18–50 kg/m^2). Briefly, a semiquantitative FFQ developed for the Study of Health and Risk in Ethnic Groups study (30) was used to assess maternal dietary intake on one occasion after recruitment

at about midpregnancy by asking the participants to answer questions related to their usual eating habits during the last year. The complete FFQ was analyzed by using a database linked to the Canadian Nutrient File. Nutrient composition was calculated as previously described (31), excluding records for which the FFQ was 50% incomplete or with implausible dietary intakes (<500 or >4,500 kcal/day). The use of supplements was also assessed as part of the FFQ, which included a separate supplemental questionnaire for cod liver or halibut oil supplement usage. The DQI score used to classify the nutritional status of pregnant women into healthy and unhealthy eating categories was based on reported daily servings of foods from 36 harmonized food groups as described previously (29). This aggregate score reflects differences in the overall nutritional quality of foods consumed, which was based on the sum of the daily number of servings of healthy and nutrient-rich foods (e.g., fermented dairy, fish and seafood, vegetables, fruits, whole grains, nuts and seeds) minus the daily number of servings of unhealthy and processed foods (e.g., processed meats, refined grains, fries, snacks, sweets and sweet drinks). A positive DQI score signifies consumption of greater amounts of healthy than unhealthy foods, and vice versa, for a negative DQI score. In this study, 25 participants were selected from the top 10th percentile to form the "good diet" group, and the bottom 10th percentile of the cohort comprised the "poor diet" group, as summarized in supplemental Fig. 1.

High-dose ω-3 PUFA supplementation in women and serum NEFA trajectories

Serum NEFAs were analyzed using fasting serum samples from a clinical intervention trial in 2017 and 2018 investigating the effect of ω-3 PUFA supplementation from FO on attenuating skeletal muscle atrophy following leg immobilization (28). The trial was registered at the US National Library of Medicine (https://clinicaltrials.gov/) as NCT03059836. Briefly, a cohort of healthy young women with a mean age of 22 years (range: 19–31 years) and BMI of 24 kg/m^2 (range: 18–26 kg/m^2) were recruited from the greater Hamilton area to participate in a randomized, double-blind, placebo-controlled intervention study. Participants received either the active treatment arm of a high-dose ω-3 PUFA from FO (3.0 g EPA and 2.0 g DHA daily; $n = 9$) or a control based on an isoenergetic and volume equivalent sunflower oil daily ($n = 9$). Serum samples in the resting fasted state were collected from participants at baseline and at 28, 42, and 56 days following the initiation of the intervention. These samples were then stored at −80°C. Participants taking FO supplements within 6 months of the study were excluded, two participants had missing residual serum samples from the original study, and one participant was reported to be noncompliant to leg immobilization intervention (28) and possibly dietary adherence. A comprehensive analysis of serum NEFAs in this work was compared with FA concentrations reported for the erythrocyte membrane PL fraction measured using a validated protocol based on GC with flame-ionization detection (28).

Validated method protocol for serum NEFAs and total FA analysis by MSI-NACE-MS

NEFAs from fasting serum samples collected from young women in both studies, along with standard human serum (Sigma-Aldrich) used as a QC specimen, were analyzed using a previously validated protocol for rapid serum NEFA determination based on MSI-NACE-MS that included an extensive intermethod comparison to GC-MS (26). In all cases, frozen serum aliquots were thawed once slowly on ice prior to analysis. Briefly, protein-bound circulating FAs were extracted from serum in acidified (3.7% vol of 1.0 M HCl) methyl-*tert*-butyl ether (MTBE):methanol (5:1; v/v) containing butylated hydroxytoluene (0.01% vol) as an antioxidant additive during sample processing and a deuterated analogue of myristic acid, 14:0-d27, as a recovery standard. Following vigorous shaking, phase separation was then induced by the addition of deionized water, and samples were then centrifuged to sediment protein at the bottom of the vial (at 3,000 *g* at 4°C for 30 min) followed by a biphasic water and ether (top) layer. A fixed volume (200 µl) was collected from the upper MTBE layer into a new vial and then dried under a gentle stream of nitrogen gas at room temperature. Dried serum extracts were then stored at −80°C and at the time of analysis reconstituted in 25 µl acetonitrile-isopropanol-water (70:20:10, v/v/v) with 10 mM ammonium acetate and 50 µM deuterated stearic acid, 18:0-d35 as an internal standard. For the analysis of total (hydrolyzed) serum FAs, acid-catalyzed hydrolysis was performed using 2.5 M sulfuric acid and 0.01% vol butylated hydroxytoluene in toluene followed by incubation at 80°C for 1 h (26). MTBE extraction was then carried out to recover total serum FAs similar to the protocol outlined for serum NEFAs. An Agilent 6230 TOF mass spectrometer with a coaxial sheath liquid ESI ionization source equipped with an Agilent G7100A capillary electrophoresis (CE) unit was used for all experiments (Agilent Technologies Inc.). An Agilent 1260 Infinity isocratic pump was used to deliver 80% vol methanol with 0.5% vol ammonium hydroxide at a flow rate of 10 µl/min using a CE-MS coaxial sheath liquid interface kit. Separations were performed on bare fused-silica capillaries with an internal diameter of 50 µm, outer diameter of 360 µm, and total length of 95 cm (Polymicro Technologies Inc.). The applied voltage was set to 30 kV at 25°C for CE separations together with an isocratic pressure of 20 mbar (2 kPa). The background electrolyte was 35 mM ammonium acetate in 70% vol acetonitrile, 15% vol methanol, and 5% vol isopropanol, with an apparent pH of 9.5 adjusted by the addition of 12% vol ammonium hydroxide. Serum extracts were injected hydrodynamically at 50 mbar (5 kPa) alternating between 5 s for each sample plug and 40 s for the background electrolyte spacer plug for a total of seven discrete samples analyzed within 30 min for a single run (26, 32). Repeat QC samples introduced in a randomized position for each MSI-NACE-MS run were analyzed for NEFA ($n = 8$) and total FA analysis ($n = 8$) to assess the technical precision of the method. All FA extracts were analyzed directly by MSI-NACE-MS without chemical derivatization when using negative ion mode detection at 3,500 V with full-scan data acquisition (m/z 50–1,700), which allows for comprehensive screening of 24 FAs consistently measured in human serum extracts.

Data processing and statistical analyses

MSI-NACE-MS data were analyzed using Agilent Mass Hunter Workstation software (Qualitative Analysis version B.06.00, Agilent Technologies, 2012). Molecular features were extracted in profile mode within a 10 ppm mass window, and serum NEFAs were annotated on the basis of their characteristic m/z corresponding to their intact deprotonated molecular ion $[M\text{-}H]^-$ and relative migration time (RMT) reflecting the electrophoretic mobility for anionic FAs. Extracted ion electropherograms were integrated after smoothing using a quadratic/cubic Savitzky-Golay function (15 points), and integrated peak areas and apparent migration times were normalized to stearic acid-d35 to determine relative peak area and RMT. Least-squares linear regression analysis for external calibration curves and control charts were performed using Excel (Microsoft Office). Principal component analysis was used for data visualization (i.e., data trends/outlier detection) when comparing the technical variance of QC samples with the overall biological variance of serum NEFA concentrations between subjects when using MetaboAnalyst version 4.0 (www.metaboanalyst.ca) (33). For multivariate analysis for data visualization and univariate analysis comparing maternal diet subgroups, data were normalized using a generalized log transformation and autoscaled with a false discovery rate correction applied for

multiple hypothesis testing. A QC-based batch-correction algorithm was also performed to correct for long-term signal drift in ESI-MS for robust serum NEFA determination using an algorithm available in the *R* project for statistical computing (34). Normality tests, Pearson and Spearman rank correlations, Student's *t*-test, and nonparametric statistical analysis (Mann-Whitney *U* test) were performed using SPSS version 18 (IBM), whereas MedCalc version 12.5.0 (MedCalc Software) was used to generate boxplots. To assess the validity of serum NEFAs and total FAs against reported dietary intakes of fish, full-fat dairy, and total fiber, as well as the DQI score, we used a Spearman's rank correlation coefficient (r) for nontransformed data. A correlation coefficient of r = 0.1–0.3 was considered a small effect, r = 0.3–0.5 a moderate effect, and $r > 0.5$ a large effect. To minimize error by accounting for EPA and DHA sources from both the diet and supplementation, a total daily ω-3 PUFA servings score (total ω-3) was devised, calculated from the FFQ as the sum of EPA and DHA from self-reported dietary intake (g/day) and self-reported supplement use (g/day). Multiple regression models were constructed for log-transformed measured serum NEFA concentrations to account for potential confounding variables (i.e., BMI, cholesterol, and HDL) that were different ($P < 0.05$) between the two dietary subgroups. Circulating NEFAs are reported in terms of absolute molar concentrations (μM) as a standardized way of enabling data comparisons independent of analytical platform, sample workup protocols, and total number of FAs measured. A two-way between- and within-mixed-model ANOVA (treatment; time) was used for assessing the impact of high-dose FO supplementation to alter circulating NEFA concentrations in healthy/nonpregnant women compared with a placebo control. A Pearson correlation coefficient for non-transformed data was used to test the association between serum NEFAs and FAs from the hydrolyzed erythrocyte PL fraction.

RESULTS

High-throughput serum NEFA determination by MSI-NACE-MS

Overall, 24 serum FAs (ranging from 9:0 to 24:1) were reliably measured as their NEFAs and/or total hydrolyzed FAs by MSI-NACE-MS from serum ether extracts (>95%) with acceptable technical precision [coefficient of variation (CV) <15%] when using standard serum for QC as summarized in supplemental Table S1. Serum FAs were analyzed after normalization of their ion responses to a single deuterated internal standard (18:0-d35) added to all samples, and most circulating FAs were quantified in terms of their absolute concentration (μM) using an external calibration curve. Each run consisted of a serial injection of six randomized serum samples together with a QC as shown in **Fig. 1A** for representative serum NEFAs annotated by their characteristic *m/z*:RMT. Anionic FAs are resolved on the basis of differences in their electrophoretic mobility (i.e., carbon-chain length, degrees of unsaturation) that migrate after the electroosmotic flow away from major neutral/zwitter-ionic lipids (i.e., TGs, PLs, cholesterol) when using an alkaline nonaqueous buffer system and detected as their intact molecular ion $[M\text{-}H]^-$ under negative ion mode using a coaxial sheath liquid interface (26); however, geometric isomers for certain FAs are not baseline resolved. Figure 1B highlights that a larger biological variance was evident for fasting serum NEFAs (mean CV = 62%) compared with corresponding total FAs from serum lipid

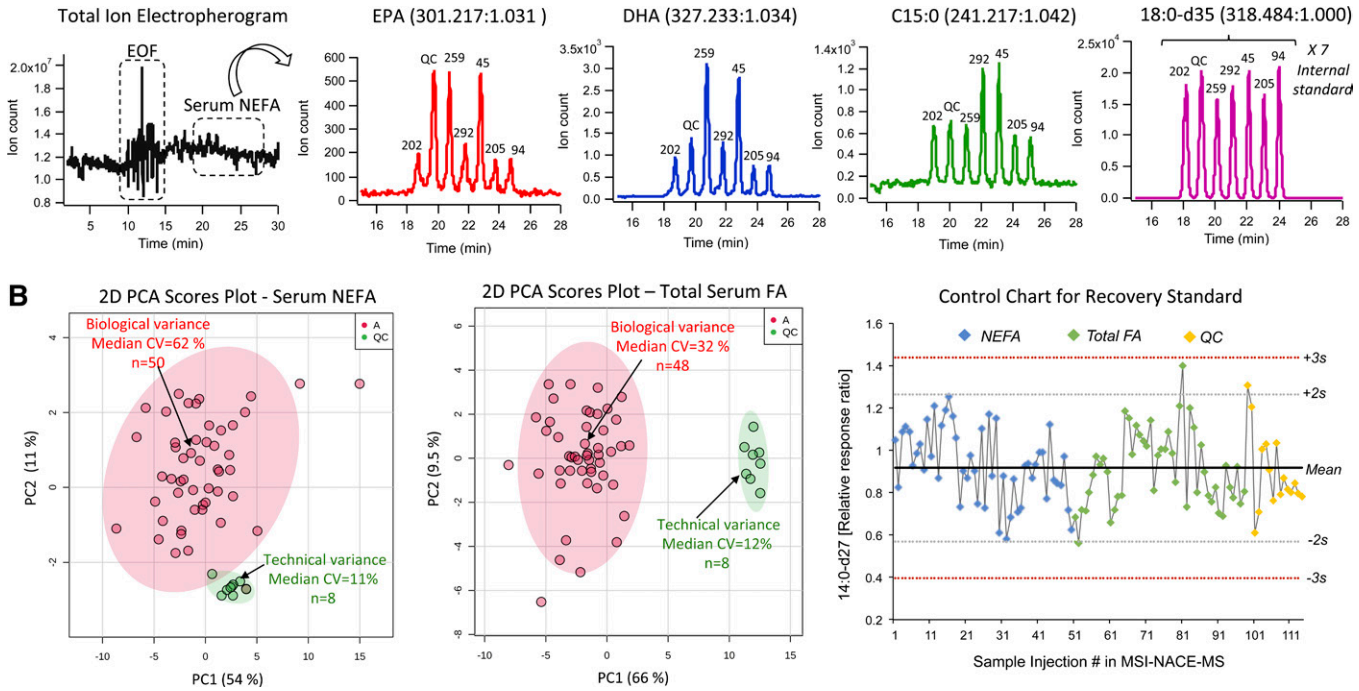

Fig. 1. A: Multiplexed separations of FAs from serum extracts for the assessment of dietary fat intake in pregnant women using MSI-NACE-MS under negative ion mode detection. This method relies on a serial injection of six randomized samples and a QC within each run to enhance sample throughput, where traces depict a total electropherogram, and a series of extracted ion electropherograms for representative serum NEFAs annotated by their characteristic *m/z*:RMT, including a deuterated internal standard for data normalization. B: Unsupervised multivariate data analysis using principal component analysis depicting the biological variance from 24 FAs as their NEFAs or total hydrolysates, compared with the technical variance from repeat QC, including a control chart for a recovery standard added to all processed serum samples.

hydrolysates (mean CV = 32%) in pregnant women ($n = 50$). Good technical precision was also confirmed on the basis of a repeat analysis of QC samples (mean CV = 12%; $n = 8$), and a control chart for a recovery standard (14:0-d27) demonstrated reliable long-term performance with few samples (~2.6%; $n = 114$) exceeding warning limits (±2 SDs). Previous method validation studies demonstrated good mutual agreement for serum FA determination when using MSI-NACE-MS compared with GC-MS (26), which is optimal for higher-throughput NEFA screening (<4 min/sample) without precolumn chemical derivatization, lipid fractionation, and/or hydrolysis artifacts as heating is avoided during sample workup. Only about 5% to 6% of total PUFAs are protein-bound NEFAs (e.g., DHA, EPA, and AA) unlike other FAs that are not extensively esterified into blood lipids, such as lauric acid (12:0) and myristic acid (14:0), as summarized in supplemental Table S1.

Serum ω-3 PUFA status reflects differences in diet quality and habitual fish intake

Anthropometric and clinical data from second-trimester pregnant women classified by their contrasting habitual diets are summarized in **Table 1**. Overall, 50 women were selected from eligible FAMILY participants (supplemental Fig. S1), reflecting healthy (median DQI score = 12.0 ± 1.9) and nonhealthy (median DQI score = −9.1 ± 3.0) maternal eating patterns, respectively. In this study, associations of serum NEFA concentrations to self-reported dietary intake from a standardized FFQ were evaluated rather than maternal health or birth outcomes. As expected, prepregnancy BMI was lower in the healthy eating diet subgroup, but there were no differences in age and weight gain during pregnancy, fasting glucose concentrations, hemoglobulin glycation, serum triglycerides, and LDL; however, total and HDL cholesterol were modestly lower in the poor diet quality maternal group ($P < 0.02$). Importantly, total fiber intake and daily fish/seafood servings were significantly higher in the healthy eating diet group ($P < 0.0001$) unlike full-fat dairy intake because it was not used as a variable in the DQI score for participant selection. As expected, pregnant women consuming a healthy diet had consistently higher circulating concentrations of ω-3 PUFAs, namely DHA and EPA, in terms of their serum NEFAs and total FAs compared with the poor diet quality maternal subgroup (**Fig. 2A**, B). Moreover, moderate correlations ($r = 0.3$–0.5; $P < 0.05$) were measured between serum EPA, DHA, and their sum [EPA + DHA] relative to the DQI score, as well as total ω-3 PUFAs from the FFQ based on daily average intakes of EPA and DHA estimated during pregnancy from both dietary sources and FO supplement use, as highlighted in **Table 2**. In fact, the strongest correlation to self-reported total intake of ω-3 PUFAs was serum EPA either as its NEFA or total FA with $r = 0.46$ and 0.50 ($P < 0.001$), respectively. As for the correlation of circulating DHA with total ω-3 PUFA intake, it was found to be higher for NEFA ($r = 0.40$; $P = 0.0040$) compared with total serum FA hydrolysate ($r = 0.33$; $P = 0.024$). Only 4 of 50 women were reported to be taking FO supplements during pregnancy, and these women had the highest circulating NEFA concentrations for EPA. Supplemental Tables S2 and S3 summarize results from the linear regression model based on measured EPA and DHA concentrations as a function of the DQI score and total ω-3 PUFAs with adjustments for covariates between both diet groups, namely BMI, total cholesterol, and HDL. Overall, correlations remained significant ($P < 0.05$) after adjustments for BMI and total cholesterol, as well as HDL in most cases. The scatterplot in Fig. 2C illustrates the positive correlation ($r = 0.43$; $P = 0.0020$) between measured concentrations for [EPA + DHA] compared with self-reported ω-3 PUFA intake (g/day). A histogram is also provided in Fig. 2C for circulating EPA as its NEFA, which had a median serum concentration of 1.64 μM. A correlation analysis (supplemental Table S4) between serum NEFAs and total FAs also demonstrated a much stronger association for EPA ($r = 0.57$; $P = 2.0 \times 10^{-5}$) compared with DHA ($r = 0.29$; $P = 0.049$), highlighting the unique attribute of this low-abundance circulating ω-3 PUFA.

Serum odd-chain/saturated FAs reflect full-fat dairy and total fiber intake

Certain saturated FAs as their serum NEFAs and/or total FA hydrolysates were correlated with self-reported intake of

TABLE 1. Anthropometric and clinical characteristics of a cross-section of second-trimester pregnant women with contrasting dietary patterns from the FAMILY study

Parameter	Poor Diet Group ($n = 25$)	Good Diet Group ($n = 25$)	P
Age (years)	31.3 ± 5.2	33.7 ± 5.7	0.14
Prepregnancy BMI (kg/m^2)	29.3 ± 7.4	24.6 ± 5.3	0.011
Gestational weight gain (kg)	14.8 ± 6.9	14.1 ± 4.4	0.69
Total cholesterol (mmol/l)	6.0 ± 1.2	6.9 ± 1.0	0.015
LDL (mmol/l)	3.1 ± 1.0	3.5 ± 1.3	0.32
HDL (mmol/l)	1.8 ± 0.4	2.2 ± 0.5	0.006
TGs (mmol/l)	2.2 ± 0.8	2.3 ± 0.9	0.76
Fasting blood glucose (mmol/l)	4.4 ± 0.4	4.4 ± 0.5	0.69
HbA1c	0.05 ± 0.01	0.05 ± 0.003	0.65
Diet quality index score	−9.1 ± 3.0 (−16.5 to −6.2)	12.0 ± 1.9 (10.8–17.4)	<0.0001
Fish/seafood (servings/day)	0.1 ± 0.1	0.3 ± 0.2	<0.0001
Total [EPA+ DHA] (g/day)	0.07 ± 0.06	0.39 ± 0.50	<0.0001
Total fiber (g/day)	18.3 ± 8.3	34.2 ± 7.6	<0.0001
Full-fat dairy (servings/day)	1.2 ± 1.1	1.6 ± 1.4	0.36

Data are presented as mean ± 1 SD. Statistical comparisons assuming equal (*t*-test) or unequal variance (Welch's *t*-test) or nonparametric Mann-Whitney tests were performed as appropriate. $P < 0.05$ was considered significant.

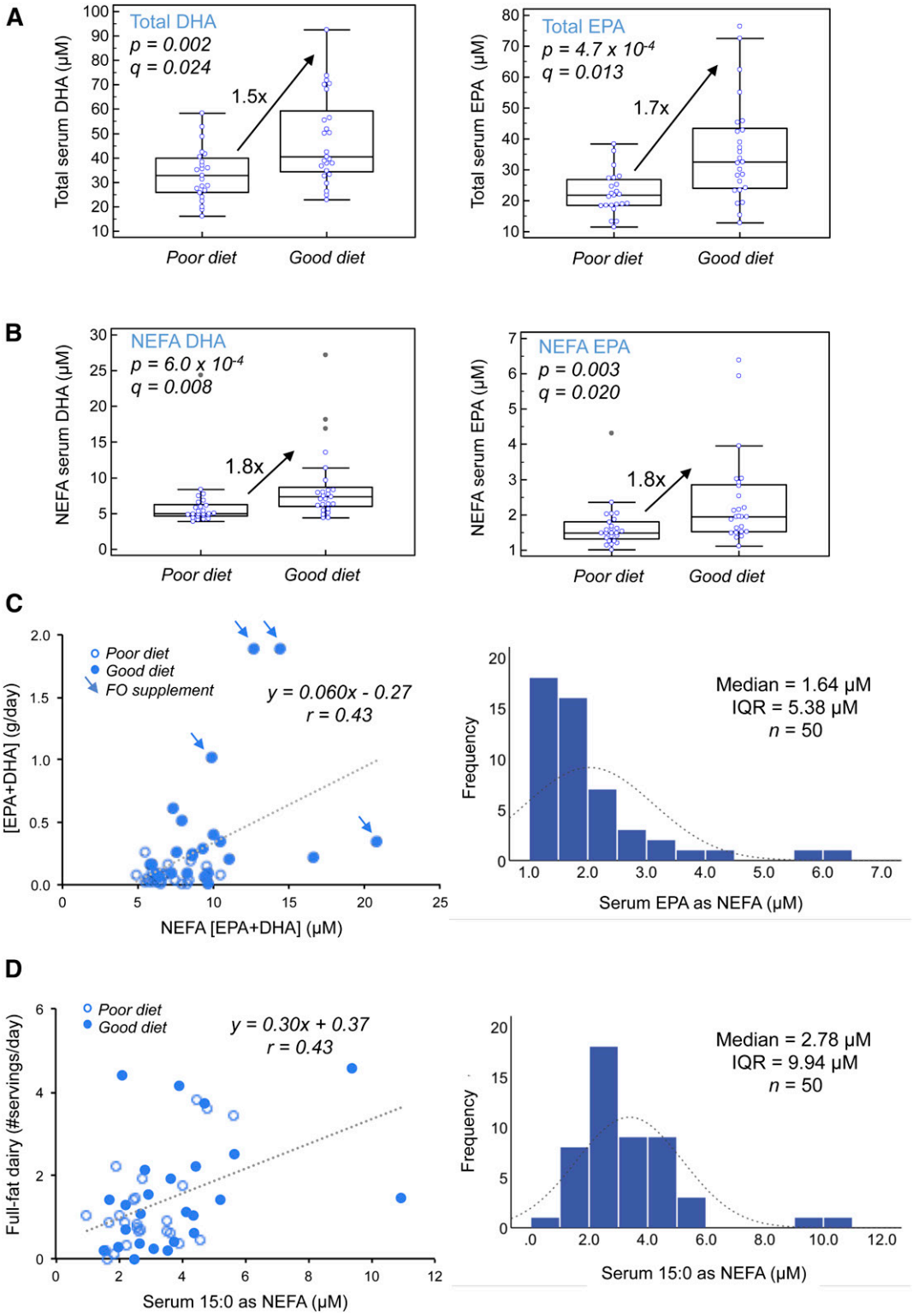

Fig. 2. Box and scatter plots for ω-3 PUFAs in serum as (A) total hydrolyzed FAs and (B) NEFAs among pregnant women with contrasting diets based on a DQI score using a univariate Mann-Whitney test ($P < 0.05$) and after false discovery rate adjustments ($q < 0.05$). C: Scatter plot (left) showing the correlation of serum [EPA + DHA] measured as their NEFAs by MSI-NACE-MS with self-reported total daily intake of ω-3 PUFAs from a FFQ and histogram (right) showing the concentration distribution of EPA as its NEFA. D: Scatter plot (left) showing the correlation of 15:0 as its NEFA with daily servings of full-fat dairy from a FFQ and histogram (right) showing the concentration distribution of 15:0 as its NEFA.

TABLE 2. Spearman rank correlation coefficients between serum ω-3 PUFA concentrations measured as their NEFA or total (hydrolyzed) FA fraction compared with the diet quality index score, fish/seafood daily servings, and total ω-3 PUFA intake in pregnant women ($n = 50$) with contrasting diets from the FAMILY study

	Diet Quality Index Score[a]		Fish/Seafood (servings/day)		Total ω-3 PUFAs[b] (g/day)	
Serum FAs	*r*	*P*	*r*	*P*	*r*	*P*
NEFAs						
EPA (20:5*n*–3)	0.29	0.043*	0.45	0.0010**	0.46	0.0010**
DHA (22:6*n*–3)	0.38	0.0060**	0.36	0.011*	0.40	0.0040**
[EPA + DHA]	0.36	0.010**	0.40	0.0040**	0.43	0.0020**
Total FAs						
EPA (20:5*n*–3)	0.37	0.0090**	0.47	0.0010**	0.50	0.00030**
DHA (22:6*n*–3)	0.28	0.052	0.29	0.045*	0.33	0.024*
[EPA + DHA]	0.40	0.004**	0.43	0.0020**	0.46	0.0010**

*$P < 0.05$ and **$P < 0.01$.

[a]Aggregate score reflecting differences in nutritional quality of foods consumed based on the sum of the daily number of servings of healthy/nutrient-rich foods (e.g., fermented dairy, fish/seafood, vegetables, fruits, whole grains, nuts/seeds) minus the daily number of servings of unhealthy/processed foods (e.g., processed meats, refined grains, fries, snacks/sweets).

[b]Daily average servings of [EPA + DHA] intake estimated from the FFQ, including diet (fish/seafood) and FO supplement use.

full-fat dairy (**Table 3**). For instance, serum pentadecanoic acid (15:0) as its NEFA had the strongest association with full-fat dairy intake ($r = 0.43$; $P = 0.0020$), whereas heptadecanoic acid (17:0) was not significant ($r = 0.21$; $P = 0.15$). However, 17:0 from total hydrolyzed serum had a weak correlation to full-fat dairy ($r = 0.29$; $P = 0.043$). In this case, total 17:0 was also associated with daily fiber servings ($r = 0.29$; $P = 0.050$), including both soluble ($r = 0.38$; $P = 0.008$) and insoluble fiber ($r = 0.31$; $P = 0.034$) fractions. In addition, serum myristic acid (14:0) showed a similar outcome as 15:0 as its NEFA ($r = 0.30$; $P = 0.034$) and total FA ($r = 0.35$; $P = 0.016$), albeit with a more moderate correlation to full-fat dairy intake. The scatterplot in Fig. 2D highlights the positive correlation of fasting serum 15:0 as its NEFA to the self-reported daily intake of dairy products (servings/day). NEFAs 14:0 and notably 15:0 are selective biomarkers of dairy fat because they were not correlated to either low-fat or fermented dairy intake. Moreover, circulating NEFA 15:0 status did not differentiate dietary subgroups of pregnant women from FAMILY ($P = 0.36$), as they had similar consumption patterns for full-fat dairy (Table 1) with a median serum concentration of 2.78 μM for 15:0 as its NEFA.

Dietary intervention study in women: FO supplementation and serum NEFA trajectories

In this study, serum NEFAs were analyzed in fasting serum samples from 18 young women collected at 4 time points over a 56 day intervention period, including baseline. For the active treatment arm, there was a mean 2.5-fold increase in serum NEFA concentrations for [EPA + DHA] from baseline after 28 days following high-dose FO supplementation compared with the placebo group; however, there were no further changes in serum concentrations of EPA, DHA, or [EPA + DHA] at later sampling times (42 and 56 days) when using a two-way mixed-model ANOVA ($P = 0.012$) as shown in **Fig. 3A**. As expected, temporal concentrations did not change for other serum NEFAs measured by MSI-NACE-MS either within subjects or between treatment arms at all time points (**Table 4**). This includes circulating linoleic acid and oleic acid despite being major constituents of sunflower oil consumed in the placebo group (Fig. 3B); these two major FAs in the circulation are highly abundant in numerous other food sources in the diet. Overall, changes in serum EPA as its NEFA was found to be more sensitive to FO supplementation compared with DHA or [EPA + DHA], which also was able to

TABLE 3. Spearman rank correlation coefficients between serum FAs measured as their NEFAs or total (hydrolyzed) FA fraction compared with full-fat dairy daily servings and total fiber in pregnant women ($n = 50$) with contrasting diets from the FAMILY study

	Full-Fat Dairy[a] (servings/day)		Total Fiber[b] (g/day)	
Serum FAs	*r*	*P*	*r*	*P*
NEFAs				
Myristic acid (14:0)	0.30*	0.034	0.040	0.76
Pentadecanoic acid (15:0)	0.43**	0.0020	0.12	0.39
Heptadecanoic acid (17:0)	0.21	0.15	−0.26	0.86
Total FAs				
Myristic acid (14:0)	0.35*	0.016	0.11	0.48
Pentadecanoic acid (15:0)	0.33*	0.023	0.22	0.14
Heptadecanoic acid (17:0)	0.29*	0.043	0.29*	0.050

*$P < 0.05$ and **$P < 0.01$.

[a]Daily average servings of full-fat dairy products estimated from the FFQ, including intake of cream of any kind, whole milk, milk, cottage/ricotta cheese, cream cheese, sour/whipping cream, and full-fat cheese.

[b]Daily average intake of total fiber from various dietary sources estimated from the FFQ.

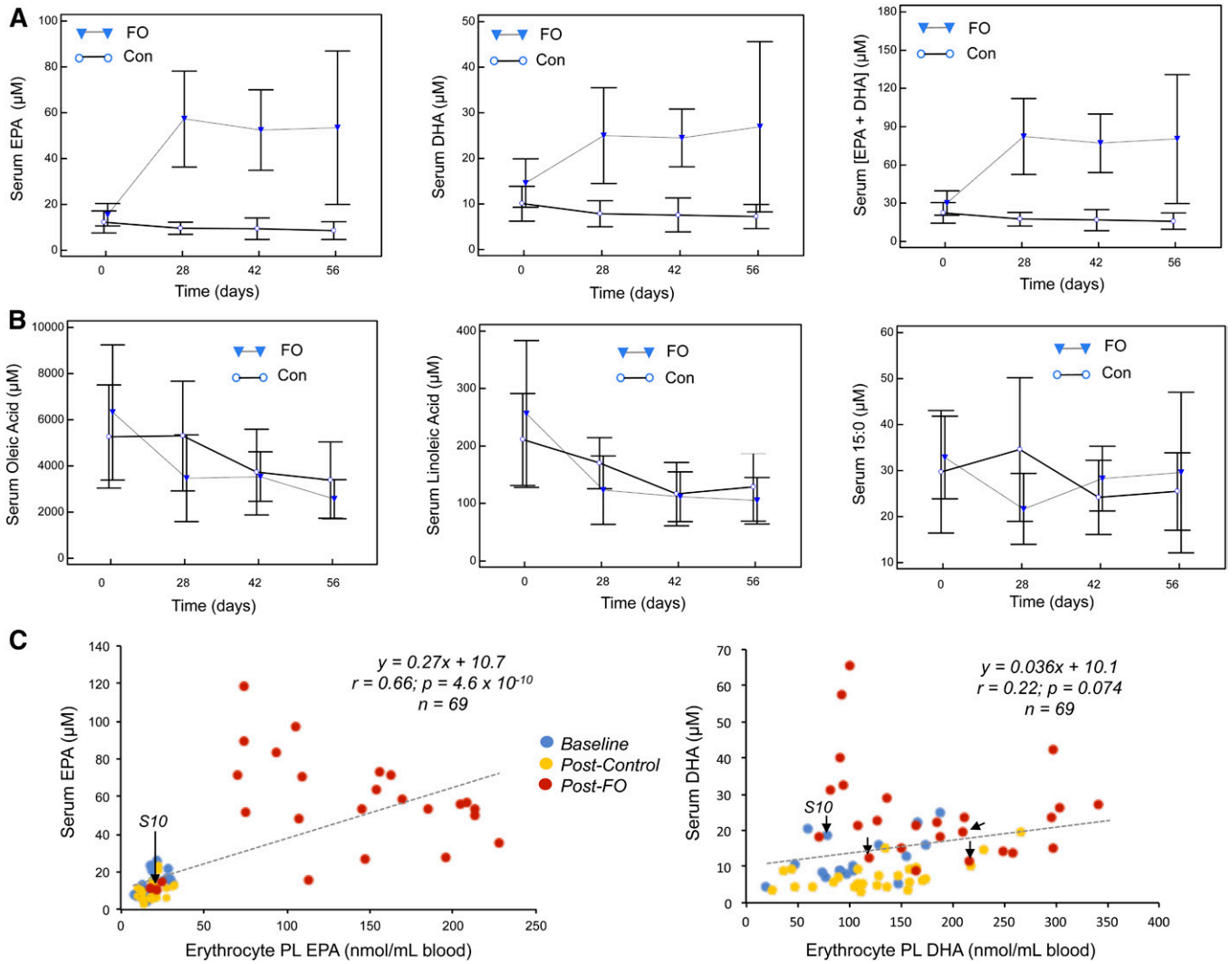

Fig. 3. Graphs depicting dynamic changes in serum NEFA concentrations in the intervention study in young women for the high-dose FO supplementation group ($n = 9$) compared with sunflower oil (Con) ($n = 9$) based on means ± 2 SDs, including (A) responsive EPA, DHA, and [EPA + DHA] in contrast to (B) nonresponsive serum NEFA controls, including oleic acid, linoleic acid, and pentadecanoic acid. The former two FAs are major constituents in the sunflower oil used that did not change in the control group. C: Scatter plots showing a strong linear correlation ($r = 0.66$) between EPA concentrations as its serum NEFA compared with corresponding erythrocyte PL fraction at all time points ($n = 69$), unlike the much weaker correlation ($r = 0.22$) for DHA concentrations. Serum EPA was also more sensitive in detecting dietary nonadherence to FO supplementation, as indicated by the arrows for one participant (S10), compared with DHA as its NEFA.

readily detect a nonadherent participant (S10) to FO supplementation previously reported not to be compliant with leg immobilization protocols (28). These outcomes are likely a result of the lower concentrations of EPA in the circulation and the higher dosage of EPA (∼3 g or 50% higher than DHA) used in FO supplement relative to DHA. Independent measurements available for FAs from erythrocyte membrane PL hydrolysates showed a mean fold change in [EPA + DHA] concentration of 2.6 from baseline that was consistent with serum NEFA measurements. Further exploration of the underlying relationship between these two distinctive blood lipid pools revealed a strong correlation only for EPA ($r = 0.66$; $P = 4.6 \times 10^{-10}$) at all time points ($n = 69$) when comparing concentrations from NEFA (protein-bound) and erythrocyte (membrane-bound) PL fractions in matching blood samples unlike DHA ($r = 0.22$; $P = 0.074$), as depicted in Fig. 3C. A moderate correlation for DHA was only evident when comparing baseline and control cases ($r = 0.35$; $P = 0.015$; $n = 44$) after excluding data from the ω-3 PUFA treatment arm postsupplementation. No associations were found for other FAs analyzed from these two blood fractions when using validated MSI-NACE-MS and GC-flame ionization detection methods.

DISCUSSION

For the first time, we report that fasting serum NEFAs have promising utility for monitoring dietary fat intake and FO supplementation in women. Various blood fractions have been used for FA determination in nutritional studies (35, 36), ranging from circulating lipid pools involved in transport (e.g., NEFA, serum PL fraction) and

TABLE 4. Fasting serum NEFAs identified using a two-way mixed-model ANOVA with repeated time points for within-subject effects between high-dose FO supplementation and control (sunflower oil) groups

	Within-Subject Effects				Between-Subject Effects			
Serum FAs	*F*	*P*[a]	Effect Size[b]	Study Power	*F*	*P*[a]	Effect Size[b]	Study Power
EPA	8.36	0.0020	0.39	0.99	22.2	0.0004	0.63	0.99
[EPA + DHA]	5.59	0.0030	0.30	0.92	19.0	0.0010	0.59	0.98
DHA	3.54	0.023	0.21	0.74	13.1	0.0030	0.50	0.92
Myristic acid	1.93	0.14	0.13	0.46	1.5	0.24	0.10	0.21
Pentadecanoic acid	1.28	0.30	0.090	0.31	0.0	0.95	0.00	0.05
Heptadecanoic acid	0.28	0.84	0.020	0.10	0.26	0.62	0.02	0.080
Linoleic acid	0.34	0.80	0.030	0.11	1.5	0.24	0.11	0.21
Oleic acid	0.50	0.68	0.040	0.13	1.9	0.19	0.13	0.25

[a] Mixed-model ANOVA significant at the $P < 0.001$ level for EPA and DHA only for within- and between-subject effects, where data sphericity was assumed/satisfied using Mauchly's test of sphericity.
[b] Based on partial eta squared.

cellular function (e.g., erythrocyte or platelet membrane) to long-term storage (e.g., adipose tissue TGs) (37); however, reports on serum NEFAs as biomarkers of dietary fat have been sparse, likely due to technical challenges in limiting background lipid hydrolysis even under mild-reaction conditions for the preparation of FA methyl esters prior to GC analysis (38). GC methods offer excellent selectivity for the resolution of FAs and some geometric/positional isomers but require longer analysis times (>20 min), even when using optimal column and elution conditions, for comprehensive FA determination (39). Alternatively, rapid serum NEFA screening can be achieved by an enzymatic-based colorimetric assay, yet this less selective method is prone to bias with discordant results compared with LC-MS methods (40). In addition, separation-free, direct-infusion, high-resolution MS (41) or multiplexed chemical isotope labeling with LC-MS (42) offer greater sample throughput, but these approaches are better suited for analyzing total hydrolyzed FAs from serum/plasma after sample processing. In this work, fasting serum NEFAs were directly analyzed using a multiplexed separation platform based on MSI-NACE-MS (26), which offers a rapid method for assessing complex dietary patterns associated with a health-promoting Prudent diet (9). Equivalent or better correlations to self-reported intake of fish/seafood, full-fat dairy, as well as FO supplementation were achieved for certain serum NEFAs compared with their corresponding total FAs from serum hydrolysates or erythrocyte membrane PL fractions. Indeed, there are conflicting reports on the exact relationship of fasting blood NEFAs and FAs from adipose tissue (19, 43, 44), where circulating NEFAs may serve as a virtual surrogate for tissue biopsies and thus more accessible biomarkers of habitual diet that also respond to acute changes in the intake of seafood and dairy products (45).

Overall, our results from the cross-sectional study are consistent with a subset of participants from the European Prospective Investigation into Cancer and Nutrition (EPIC) study, where EPA and DHA had moderate correlations to self-reported fish intake from the FFQ based on either total hydrolysates from the plasma PL fraction ($r = 0.33$ and 0.29, respectively) or erythrocyte-membrane PL fraction ($r = 0.29$ and 0.40, respectively) (46). In our work, correlations for fasting serum EPA and DHA as their NEFAs ranged from 0.36 to 0.46, indicating that they provide an analogous assessment of habitual fat intake without lipid fractionation and hydrolysis as required for FA determination from serum PLs (36). For the high-dose ω-3 PUFA supplementation study, our results demonstrated a relatively fast equilibration time for serum [EPA + DHA] as their NEFAs, as reflected by a mean 2.5-fold change from baseline within 28 days, which is consistent with acute changes in circulating blood lipid pools in previous FO intervention studies (37, 47, 48); this was a selective treatment effect, as no other changes were measured in other serum NEFAs from either active treatment or sunflower oil placebo arms. We hypothesize that this fast equilibration may be a consequence of the high ω-3 PUFA dosage regime used in this study (5 g/day) that is greater than the average intake of fish for Canadians (0.1–0.7 seafood meals/week) or used in commercial supplements (~1 g/day) (22, 49). Furthermore, the strong correlation between erythrocyte membrane PLs and serum EPA as its NEFA over the duration of the study, but not DHA, supports the idea that EPA is more responsive to changes in dietary patterns, which is a consistent finding in both our observational and intervention studies. This is also in agreement with reports on other plasma fractions, where EPA responds to high-dose FO supplementation and cessation within 1 week (50) despite appreciable retroconversion of EPA to DHA (51). In our work, serum EPA concentrations (median: 1.6 μM; range: 1.0–6.3 μM) were lower ($P = 0.00010$) in pregnant women than DHA (median: 6.1 μM; range: 3.8– 27 μM) as their NEFA, which was more striking compared with differences in their total serum hydrolysate ($P = 0.0010$) concentrations (Fig. 1). In addition, EPA as its NEFA was much more sensitive in detecting self-reported FO supplement use among four pregnant women, as well as acute changes in nonpregnant women, following high-dose ω-3 PUFA supplementation compared with DHA or [EPA + DHA] (Fig. 3A; Tables 2, 4) while also revealing suspected dietary nonadherence for a participant. Furthermore, serum EPA as its NEFA was strongly correlated ($r = 0.66$; $P = 4.6 \times 10^{-10}$) to independently measured erythrocyte PL membrane concentrations unlike DHA (Fig. 3C). Consequently, we propose fasting serum EPA as its NEFA as a robust and sensitive dietary biomarker that correlates well to long-term/habitual fish intake, as well as acute changes following FO supplementation.

This is important given expanding interests in high-dose ω-3 PUFAs (either EPA + DHA or EPA only) for the prevention of muscle atrophy (28), reduction of asthma and persistent wheezing (52), promotion of lean mass and bone growth in childhood (53), and the reduction of atherosclerotic cardiovascular disease risk in patients with hypertriglyceridemia (54). However, ω-3 PUFAs can have quite distinctive lipid compositions that affect their bioavailability; they also vary up to 10-fold in natural abundance when comparing oily fish (e.g., mackerel, salmon, sardines) to other commonly consumed lean fish (e.g., haddock, canned tuna, cod) and other seafood sources (e.g., algae, krill, prawns) (55). Interestingly, the poor diet quality subgroup of pregnant women had an estimated total ω-3 PUFAs of only 71 mg/day from the self-reported FFQ compared with 217 mg/day for the healthy eating subgroup, both of which are still below 300 mg/day DHA as recommended by the International Society for the Study of Fatty Acids and Lipids Working Group (49). This information is valuable for prenatal screening of ω-3 PUFA nutritional status and reliable monitoring of individual responses to dietary modifications or supplementation regimes for optimal maternal health.

Next, OCFAs are of special interest due to their role as promising food-specific biomarkers of full-fat dairy intake, which have also been reported to be inversely associated with type 2 diabetes risk (56, 57). Observational and intervention studies have reported that 15:0 and 17:0 are dietary biomarkers reflecting milk fat intake as measured from adipose tissue TGs, serum PLs, serum cholesteryl esters, total serum lipids, and dried blood spots (56–60); however, there are sparse reports from the analysis of serum OCFAs as their NEFAs (61). In fact, serum 17:0 does not correlate with 15:0 because 17:0 can also be endogenously synthesized via α-oxidation and generated via propionate via the action of gut microbiota on fermentable fiber (56, 57); for these reasons, 17:0 may serve as a putative biomarker of dietary fiber intake. Alternatively, adipose tissue 14:0 has been proposed as a biomarker for the long-term intake of dairy fat (60). Our results confirmed that 14:0 ($r = 0.30$; $P = 0.034$) and especially 15:0 ($r = 0.43$; $P = 0.0020$) as their NEFAs were robust dietary biomarkers of full-fat dairy intake in pregnant women (Fig. 2D, Table 4) but not skim/low-fat or fermented milk. Our results for 17:0 were inconsistent when comparing NEFAs and total FA hydrolysates, with only the latter showing a weak association with self-reported intakes of full-fat dairy and total fiber, including soluble and insoluble fiber. Further studies that incorporate microbiome analyses are needed to better elucidate the utility of OCFAs as biomarkers of fiber intake because it is a major source of biological variance. Nevertheless, our work validates the use of fasting serum NEFAs as a convenient circulating lipid pool reflecting dietary intake of oily fish and full-fat dairy without invasive adipose tissue biopsies.

Strengths of our study include the use of a rapid method based on MSI-NACE-MS for quantitative serum NEFA determination with stringent QC that was applied to two independent cohorts of women involving a validated FFQ and a placebo-controlled, high-dose ω-3 PUFA clinical trial. Serum NEFAs feature rarely in nutritional studies when relying on low-throughput GC protocols that are susceptible to background lipid hydrolysis and oxidation artifacts during sample processing. This work also compared analyses between serum NEFAs and total FA hydrolysates for the assessment of long-term/habitual fat intake during pregnancy, as well as acute/short-term changes with high-dose ω-3 PUFA supplementation in women. Rapid NEFA determination by MSI-NACE-MS also offers a convenient alternative to erythrocyte membrane PL hydrolysate analyses especially in large-scale epidemiological studies given the availability of serum or plasma in biorepositories. This study has some limitations, including the modest sample size of each cohort involving a single biological sex, and the lack of self-reported diet records in the ω-3 PUFA clinical trial. In addition, because extreme diet scores from pregnant women were selected to maximize the effect size, this might have introduced a selection bias. Likewise, we aimed at studying associations of serum NEFA concentrations with a self-reported FFQ rather than assessing health outcomes of pregnant women. Long-term stability studies for protein-bound NEFAs are also needed to rule out potential bias due to prolonged storage of frozen serum samples collected from FAMILY (>10 years). For the intervention study, sampling shorter time points (<28 days) is also needed to better assess the minimum time frame required for ω-3 PUFA equilibration that is likely both dose- and sex-dependent. In conclusion, our study introduces a rapid yet inexpensive approach for the quantification of serum NEFAs that avoids serum lipid fractionation, hydrolysis, and/or precolumn chemical derivatization procedures. This approach largely provides equivalent and in some cases superior results compared with total FA hydrolyzed from serum, as well as erythrocyte PL fractions, notably in the case of assessing circulating EPA and 15:0 as optimal NEFA biomarkers of habitual intake of fish/FO and full-fat dairy, respectively. MSI-NACE-MS is anticipated to facilitate large-scale blood-based testing of serum NEFAs with greater sample throughput, lower costs, and better long-term data fidelity than standard GC protocols. This is needed to more accurately assess dietary fat intake for evidence-based nutritional policies that promote maternal health.

Data availability statement

All processed serum FA data and deidentified clinical/dietary information for participants is available as an excel file in the supporting information section (NEFA-Serum-JLR.xlsx). This data includes original and batch-corrected RPA for NEFAs measured by MSI-NACE-MS, as well as their serum concentrations from both observational and intervention studies, including matching total serum hydrolysate and independent erythrocyte PL fraction measurements, respectively.

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